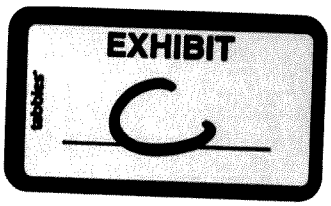
Reprinted from Nature, Vol. 335, No. 6192, pp. 726-730, 20 October 1988



Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin I

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Significant future developments in the effective treatment of inflammatory diseases may arise from non-toxic dual inhibitors of both cyclooxygenase and lipoxygenase pathways in the arachidonate cascade[1]. Inhibition of phospholipase A_2 (PLA_2) (EC 3.1.1.4), may provide such a dual action and recent research has concentrated on the role of PLA_2-inhibitory proteins as possible anti-inflammatory agents. Blastokinin[2] or uteroglobin[3] is a steroid-induced rabbit secretory protein with PLA_2-inhibitory activity. Its biochemical and biological properties have been extensively studied[4-14] and its crystallographic structure has been resolved at 1.34 Å (refs 15, 16). Lipocortins are a family of related proteins[17-22], which, it has been suggested, mediate the anti-inflammatory effects of glucocorticoids (for a review, see ref. 23). Some proteins of this group have been purified[24,25] and the complementary DNA sequences of two human lipocortins are known[25,26]. Lipocortins inhibit PLA_2 *in vitro*[17-21,24-29], although their mechanism of action is still unclear[24-29,30,31]. Recombinant lipocortin I inhibits eicosanoid synthesis in isolated perfused lungs from the guinea pig[32]. Here, we report that synthetic oligopeptides corresponding to a region of high amino-acid sequence similarity between uteroglobin and lipocortin I have potent PLA_2 inhibitory activity *in vitro* and striking anti-inflammatory effects *in vivo*.

Mature uteroglobin (UG) is an antiparallel dimer formed by two identical subunits of 70 amino acids each[15,16]. Figure 1*a* shows the alignment of the mature UG monomer[33] with lipocortins I and II (refs 25, 26). Lipocortins I and II are formed by four non-identical repeated units of about 70 amino acids each[34-36]. The program PRTALN aligns UG with a region of lipocortins I and II that approximately corresponds to the second repeat (Fig. 1*a*, I and III), the region of highest similarity between the two lipocortins. The total similarity (identities + conservative replacements) is 40% in both cases. However, the highest number of identities (19) is found between UG and lipocortin I repeat three (residues 199-275), with a total similarity of 43%. Moreover, a striking local similarity was identified between residues 40-46 of UG and residues 247-253 of lipocortin I, repeat three. In UG, this region corresponds to the C-terminal part of α-helix three (residues 32-47)[15,16]. As it is 'centered' on a relatively long region of local similarity, the alignment with repeat three is probably the most accurate. We drew four conclusions from these results. (1) There is amino-acid sequence similarity between UG and lipocortins; (2) UG is more similar to lipocortin I than to lipocortin II, although it can be aligned to the region of highest similarity between the two lipocortins; (3) compared to repeats two and three of lipocortin I, the highest density of identities and conservative replacements is in the C-terminal half of UG (residues 33-70) and particularly in α-helix three (Fig. 1*a*); (4) the region of most similarity between helix three of UG and repeat three of lipocortin I can be precisely identified as a heptapeptide spanning residues 40-46. This heptapeptide aligns with lipocortin I residues 247-253 (Fig. 1*a*).

Hydropathy profiles of UG and the corresponding regions of lipocortins are shown in Fig. 1*b* (I-IV). The similarity is evident over most of the UG sequence and particularly in the region of UG between positions 37-52. Interestingly, the hydropathy profile of porcine pancreatic PLA_2 (Fig. 1*b*, V) shows a striking similarity with that of the UG monomer and of the corresponding regions of lipocortins, even in the absence of significant sequence similarity. It has been independently suggested that lipocortin repeats and PLA_2 could have a similar three-dimensional organization[15]. Moreover, refined crystallographic data have shown that the molecular surface of UG is strikingly similar to the one of PLA_2 (ref. 16). These data may indicate that PLA_2, the UG monomer and the lipocortin repeats have a similar three-dimensional organization.

On the basis of computer analyses, we synthesized oligopeptides corresponding to the C-terminal part of UG α-helix three and tested them for PLA_2-inhibitory activity. Table 1 shows the amino-acid sequences and PLA_2-inhibitory properties of synthetic peptides derived from UG and lipocortin I. Peptide one (P1), which corresponds to the nine C-terminal amino-acid residues of α-helix three, is a very potent inhibitor of PLA_2, with ~80% inhibition at 50 nM, under the experimental conditions used. Peptide two (P2), corresponding to lipocortin I residues 246-254, is as active as P1 (Table 2). Peptide three (P3), which retains full inhibitory activity under these experimental conditions, was constructed by substituting an asparagine for a lysine residue corresponding to UG lysine 42. The removal of two amino-acid residues from the N-terminal and one residue from the C-terminal of P1 and P2 abolishes the inhibitory activity (Table 1). The 'core' tetrapeptide Lys-Val-Leu-Asp, which is common to all the active peptides, is itself inactive. Peptide seven, corresponding to UG residues 1-10 (a region of lesser similarity between UG and lipocortins) has been used as a non-specific control and is inactive as a PLA_2 inhibitor. No significant difference in PLA_2 inhibitory activity between peptides one, two and three could be detected under these experimental conditions, at peptide concentrations between 1 and 200 nM (not shown).

We further investigated the PLA_2-inhibitory properties of the peptides and their parent proteins under initial velocity conditions. Figure 2 shows that UG, P1 and P2 have comparable dose responses. However, UG had a maximal inhibitory effect at 1 nM, whereas the peptides (P1 and P2) had a similar effect at

Table 1 Amino-acid sequences of synthetic peptides and their PLA_2 inhibitory activity

Peptide	Sequence	Concentration* (M)	% PLA_2 inhibition† ± standard deviation
1	MQMKKVLDS	5×10^{-8}	81.6 ± 5.3
2	HDMNKVLDL	5×10^{-8}	89.6 ± 1.1
3	MQMNKVLDS	5×10^{-8}	86.5 ± 0.8
4	KVLD	10^{-3}	n.s.
5	MKKVLD	10^{-3}	n.s.
6	MNKVLD	10^{-3}	n.s.
7	GICPRFAHVI	10^{-3}	n.s.

PLA_2 was assayed according to Clark *et al.*[40] with minor modifications. Briefly, the reaction mixture contained 100 mM Tris HCl, *p*H 8.0, 100 mM $NaCl_2$, 1 mM $CaCl_2$, 1 mM sodium deoxycholate, 10 μM 1-stearoyl, 2-[1-^{14}C] arachidonyl phosphatidylcholine (58 mCi mmol^{-1}; Amersham) and 25 ng (approximately 36 nM) porcine pancreatic PLA_2 (Boehringer, 700 U mg^{-1}) in a total volume of 50 μl. The reaction was started by addition of the enzyme to the radioactive substrate. Reactions were run at 37 °C for 4 min. Approximately 20-30% of the substrate was hydrolysed. Appropriate controls were included with one without enzyme. To determine the inhibitory activity, PLA_2 was pre-incubated for 10 min with the putative inhibitors at 37 °C before adding the enzyme/inhibitor mixture to the substrate. There were also controls in which PLA_2 was pre-incubated with buffer. Radioactive arachidonic acid was separated from the substrate by TLC on silica plates (silica gel G, Analtech). Developing solvent was petroleum ether/ethyl ether/acetic acid (70:30:1). Iodine-stained bands co-migrating with authentic arachidonic acid were scraped and counted in a Beckman LS-9000 liquid scintillation counter. *, The concentration used was that yielding highest inhibition for active peptides; the highest concentration tested was used for inactive peptides. †, Values are means of three determinations. n.s., not significant

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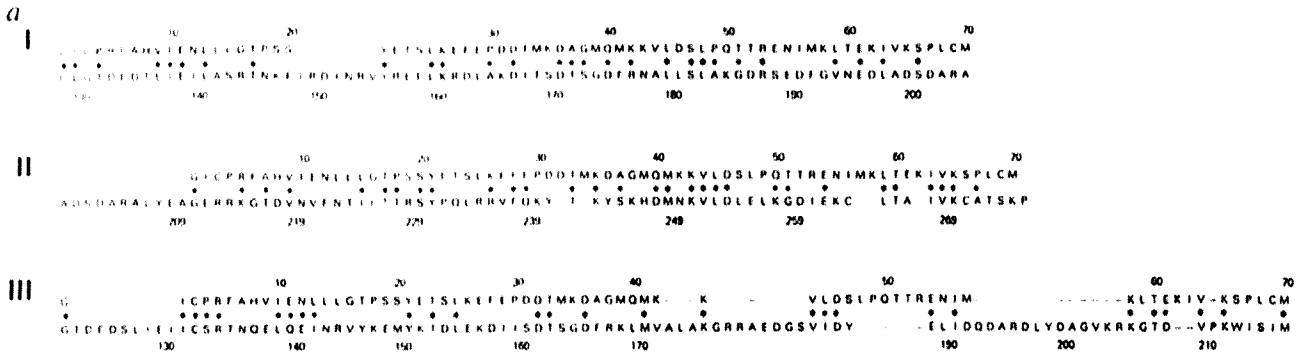
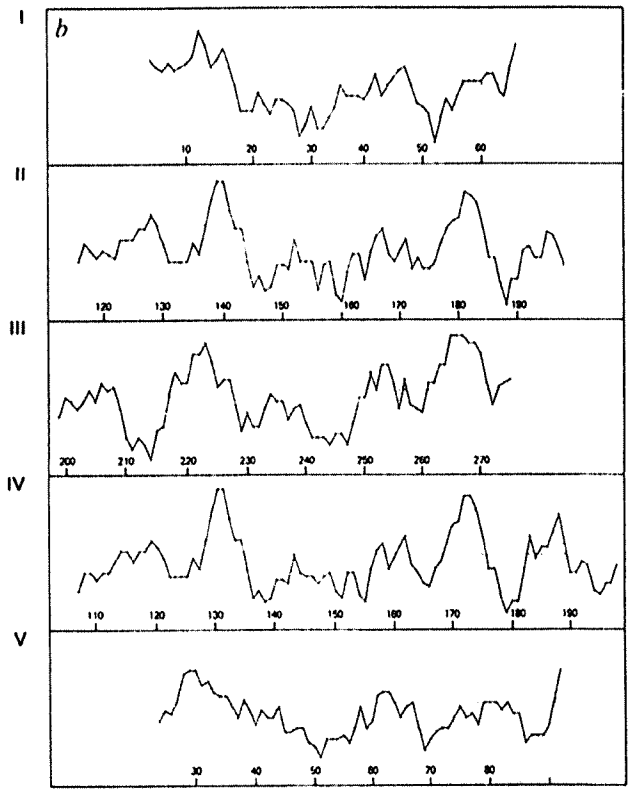


Fig. 1 *a*, Alignment of UG with lipocortin I repeat two (I), lipocortin I repeat three (II) and lipocortin II (III), obtained using the program PRTALN[44]. Identities are indicated by asterisks and conservative substitutions by dots. Conservative substitutions were added manually and are defined by pairs of residues falling into the following groups[45]: S, T, A, G, P; N, D, E, Q; R, K, H; M, I, L, V, F, Y, W. The sequence of mature UG is derived from ref. 33, the sequences of lipocortins I and II from refs 26 and 25 respectively. *b*, Hydropathy profiles of UG (I), lipocortin I repeat two (II) lipocortin I repeat three (III), lipocortin II repeat two (IV) and porcine pancreatic PLA_2, residues 24-92 (V). The profiles were obtained using a segment of seven residues. Positive peaks indicate hydrophobic regions; negative peaks indicate hydrophilic regions.



Methods. We used the following programs: PRTALN (ref. 44), FASTP (ref. 46), RDF (Ref. 46) and HYDRO. HYDRO was written by Dr C. Buckler for the NIH Molecular Biology Users Group. The program generates hydropathy profiles[47]. Similarity scores and statistical evaluations were obtained by means of FASTP and RDF. Briefly, FASTP calculates a similarity score before and after optimization of the alignment. The algorithm is similar to the one used in PRTALN, but the scoring rules are based on the 250-PAM amino acid replaceability matrix[45,46]. 'Rare' amino-acid matches get higher scores than 'common' amino-acid matches. Conservative substitutions also get positive scores, whereas 'unlikely' substitutions and mismatches get a negative score. RDF evaluates the significance of a similarity between two sequences by comparing the first sequence with n (Usually 100) random shuffles of the second. Initial and optimized scores are calculated for every comparison according to the scoring rules of FASTP. The significance is expressed as a z-value ($z = (s - m)/sd$, where s is the score of the original comparison, m is the mean score of the random-shuffled controls and sd is the standard deviation of this mean). z values between three and six are considered to be possibly significant for an evolutionary relationship, between 6 and 10 probably significant and above 10 certainly significant[46]. As a control, we used FASTP to search the 3061 sequences of the Protein Identification Resource database[48] for similarity with UG. The 111 alignments with highest scores were identified and saved. The average optimized score of these alignments is 36.09 ± 6.8. Lipocortins I and II received scores of 42 and 39, which are respectively 0.9 and 0.4 standard deviations higher than this average. We also used FASTP and RDF to calculate the similarity scores and z values obtained comparing UG to all other proteins that have been suggested to be evolutionarily related to it. These include rat prostatic steroid binding protein chain C2 and Ca^{2+} binding proteins of the calmodulin family[49,50]. Lipocortins I and II have optimized z values which are higher than all other proteins tested except prostatic steroid binding protein C2 chain precursor (not shown). When UG α-helix three alone was compared to lipocortin I repeat three initial and optimized z values were 4.24 and 3.03 standard deviations respectively.

concentrations between 4 and 8 nM. Under these conditions, both UG and the peptides had a remarkable inhibitory effect in sub-nanomolar concentrations (Fig. 2). Decreasing the enzyme concentration caused a parallel decrease in the optimum inhibitory concentrations of peptides. When P1 was pre-incubated with the lipid substrate instead of the enzyme, its inhibitory effect was drastically reduced (Fig. 2*a*). This may indicate that the inhibitory effect of P1 is exerted through an interaction with the enzyme rather than the substrate. This possibility is further supported by the observation that P1, at concentrations ranging from 1 nM to 10 μM, had no inhibitory effect on *Bacillus cereus* phospholipase C, under identical experimental conditions to those used for PLA_2 (data not shown). Figure 2*b* clearly shows that P3 under these conditions is less active than P1 and P2. This difference tends to decrease when the concentration of P3 is increased. Recombinant lipocortin I inhibits PLA_2 in this system, with an overall dose-response curve similar to that of P3. Also, lipocortin I seems to be less active than the lipocortin I-derived peptide (P2). However, this difference may be artefactual, as it has been reported that purified preparations of recombinant lipocortin I are mixtures of several disulphide-bonded forms and are contaminated with bacterial proteins[32].

Several conclusions can be drawn from these experiments. The first two residues of P1, and the last one, can be replaced by other amino acids, but not eliminated, without loss of activity. This may indicate that the length of the peptide is critical, possibly for conformational reasons; the minimum peptide length that can form two complete turns of an α-helix is eight residues. The lysine residue in P1 corresponding to lysine 42 of UG is not indispensable for the inhibitory activity. This was

Table 2 Anti-inflammatory effect of peptides 1 and 2 on carrageenan-induced rat paw oedema

Treatment	Number of animals	% Inhibition (mean ± standard deviation)
P1, 2 mg kg^{-1}	7	82.6 ± 5 ($P < 0.01$)
P1, 200 μg kg^{-1}	9	57.5 ± 7 ($P < 0.01$)
P1, 20 μg kg^{-1}	9	36.0 ± 8 ($P < 0.01$)
P1, 2 μg kg^{-1}	9	31.5 ± 9 ($P < 0.01$)
P1, 0.2 μg kg^{-1}	9	24.0 ± 5 ($P < 0.01$)
P1, 0.02 μg kg^{-1}	9	n.s. ($P > 0.05$)
P2, 2 mg kg^{-1}	8	96.2 ± 2 ($P < 0.01$)
P2, 200 μg kg^{-1}	8	30.5 ± 7 ($P < 0.01$)
P2, 20 μg kg^{-1}	8	36.3 ± 7 ($P < 0.01$)
P2, 2 μg kg^{-1}	8	23.3 ± 3 ($P < 0.01$)
P2, 0.2 μg kg^{-1}	8	20.0 ± 4 ($P < 0.01$)
P2, 0.02 μg kg^{-1}	8	12.2 ± 3 ($P < 0.05$)
DEX, 1 μg kg^{-1}	5	75.9 ± 6 ($P < 0.01$)
IND, 1 mg kg^{-1}	5	26.1 ± 4 ($P < 0.01$)
P1, 2 mg kg^{-1} + AA	4	21.1 ± 5 ($P < 0.01$)
P2, 2 mg kg^{-1} + AA	4	20.2 ± 1 ($P < 0.01$)
UG, 100 μg kg^{-1}	4	35.5 ± 7 ($P < 0.01$)
LC-1, 100 μg kg^{-1}	4	24.5 ± 1 ($P < 0.01$)
P7, 2 mg kg^{-1}	4	n.s. ($P > 0.05$)
P7, 10 μg kg^{-1}	4	n.s. ($P > 0.05$)
BSA, 2 mg kg^{-1}	4	n.s. ($P > 0.05$)
BSA, 100 μg kg^{-1}	4	n.s. ($P > 0.05$)
LSZ, 2 mg kg^{-1}	4	n.s. ($P > 0.05$)
LSZ, 100 μg kg^{-1}	4	n.s. ($P > 0.05$)

P1, peptide 1; P2, peptide 2; DEX, dexamethasone; IND, indometacin; AA, arachidonic acid; UG, uteroglobin; LC-1, lipocortin 1; P7, peptide 7; BSA, bovine serum albumin; LSZ chicken egg lysozyme; n.s., not significant. Rats (Sprague-Dawley, males, mass about 250 g) were maintained with water and food *ad libitum*, with a 12-hour-light/12-hour-darkness cycle. Animals were injected in the sub-plantar space with 1.0 mg lambda-carrageenan (Sigma, type IV) in 0.1 ml sterile saline. Inhibitors, control substances or vehicle alone were injected about 30 s after carrageenan, in a volume of 0.1 ml, to avoid non-specific interactions *in vitro* between carrageenan and inhibitors. Controls which received saline alone and saline plus vehicle were included. Peptides were dissolved in sterile 10 mM Tris, *p*H 8, because this yielded more consistent and reproducible dose responses compared to peptides dissolved in saline. Dorsoplantar paw thickness was measured with a vernier caliper[41,42] immediately before the carrageenan injection and 4 h after treatment. When AA was used, it was administered 10 μg per paw. At this dosage, AA did not cause appreciable paw swelling when administered alone, nor did it increase the swelling caused by carrageenan when administered with it (*N*, four animals per group). Inhibitory effects were assessed by comparing the dorsoplantar paw thickness of inhibitor-treated groups to that of vehicle-treated groups. The results were analysed by a one-tailed Student's *t* test for groups of unpaired observations. Significance was taken at $P < 0.05$. The statistical significance of the effects of P1 and P2 was also confirmed by one-way ANOVA[43]. Note that the dose-response curve of P1 is steeper than that of P2 and that at the highest dose tested P2 seems to have a more pronounced effect than P1.

expected, because in UG, lysine 42 is not exposed to the solvent, as it is H-bonded to the main-chain carbonyl of glycine 16 (ref. 15). However, hybrid peptide three seems to be less active than both parent peptides one and two, particularly at low concentrations, possibly because there is a decreased tendency to assume the active conformation in solution. Recombinant lipocortin I inhibits PLA_2 in our system, although with a lower activity than UG or P1, in the absence of phosphatidylserine and in the same concentration range as the enzyme. Both the presence of phosphatidylserine and a large molar excess of lipocortin over PLA_2 have been reported to be necessary for the inhibition of PLA_2 by lipocortin in assays using autoclaved *Escherichia coli* cells or extracted *E. coli* phospholipids as enzyme substrates[30,31]. The mechanism of PLA_2 inhibition by lipocortin I in our assay system is currently under investigation. The similarity in the PLA_2-inhibitory properties of P1 and purified UG support the hypothesis that P1 corresponds to an active site, or part of an active site, responsible for the PLA_2-inhibitory activity of UG.

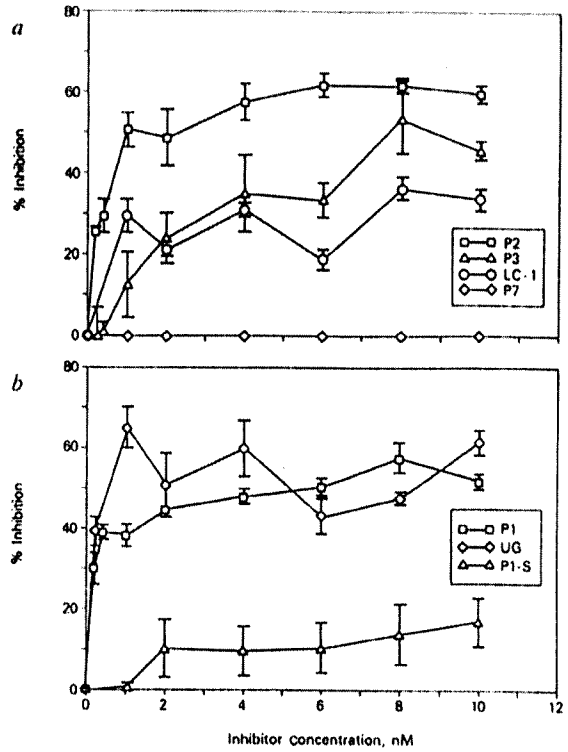


Fig. 2 Inhibition of porcine pancreatic PLA_2 by UG, lipocortin I and synthetic oligopeptides. Each point represents the mean of at least four separate determinations ± standard deviation. *a*, □, peptide 2; △, peptide 3; ○, lipocortin-I; ◇, peptide 7. *b*, □, Peptide 1; ◇, uteroglobin; △, peptide 1, pre-incubated with the lipid substrate.

Methods. Peptides were synthesized by an Applied Biosystems model 430 A peptide synthesizer. Coupling efficiency was determined by ninhydrin monitoring. Cleavage of peptides from the solid support and removal of the side-chain protecting groups were performed by an optimized hydrofluoric acid method. Peptides were purified by reverse-phase HPLC, on a C8 stationary phase. Purity was determined by HPLC and amino-acid analysis. Lyophylized peptides were stored at −20 °C under nitrogen in sealed glass vials and were redissolved immediately before use. UG was purified essentially as described[8] from the uterine flushings of rabbits pretreated with human chorionic gonadotropin. The protein seemed homogeneous as judged by SDS-polyacylamide gel electrophoresis and isoelectric focusing (*p*I 5.4) and was stored dessiccated at −20 °C in lyophilized form. PLA_2 assays were performed as described in Table 1, with 2 nM PLA_2 and a reaction time of 30 s. Under these conditions, enzyme activation is virtually instantaneous (<10 s), and is followed by an interval during which the rate of product accumulation is linear (L. Miele *et al.* manuscript in preparation). The concentration of enzyme used falls within the linear part of the velocity against enzyme concentration curve. In 30 s 0.5–1% of the substrate is hydrolysed. This allows the effects on PLA_2 activity of the accumulation of reaction product in the lipid-water interface to be minimized. Non-specific proteins (horse heart myoglobin and chicken egg lysozyme) were tested for PLA_2-inhibitory activity at concentrations between 1 nM and 10 μM and were found to be inactive. In some experiments, phospholipase C from *Bacillus cereus* (0.01 U, Boehringer, grade I) was substituted for PLA_2. In these experiments the reaction time was 4 min. TLC separation of the reaction products was carried out in the same developing system used for PLA_2, and bands co-migrating with the standard (1,2-dioleoylglycerol) were scraped and counted in a Beckman LS-9000 liquid-scintillation counter.

We tested the PLA_2-inhibitory synthetic peptides for anti-inflammatory activity *in vivo* and found that both UG-derived P1 and lipocortin I-derived P2 have very potent anti-inflammatory effect on the carrageenan-induced rat footpad oedema[37]. Table 2 shows the anti-inflammatory effects of P1 and P2 compared to those of known anti-inflammatory agents. Both P1 and

P2, when injected locally immediately after carrageenan administration, inhibited the formation of inflammatory oedema over a wide range of doses. The dose-response curves of P1 and P2 do not differ significantly, except perhaps in the range between 0.2 and 2 mg kg^{-1}. At 2 mg kg^{-1} both peptides caused a virtually complete suppression of the inflammatory response. Both parent proteins, UG and lipocortin I, when used at 100 μg kg^{-1} had anti-inflammatory activity comparable to that of a 10-fold higher dose of indometacin. The effect of both P1 and P2, at the highest dose used, is drastically reduced by a concomitant local administration of 10 μg of arachidonic acid (Table 2). This dosage of arachidonic acid had no inflammatory effect when administered alone and did not significantly increase the inflammatory effect of carrageenan when administered with it. These observations support the hypothesis that an important *in vivo* mechanism of these peptides may be the inhibition of arachidonate release from the cell membrane by PLA_2. However, our data do not rule out a minor contribution from other unknown mechanism(s) at the highest dosages of P1 and P2. Preliminary data indicate that P1 and P2 also have potent anti-inflammatory effects when administered by intraperitoneal and intravenous injection.

The evolutionary origin of PLA_2 inhibitory proteins is unknown. It has been suggested recently that lipocortins and calelectrins are derived from a 'single repeat' protein by gene duplication[38]. This protein might also be the ancestor of the UG monomer. However, we cannot determine from our data whether the similarities arose from convergent or divergent evolution. We believe that the peptides described in this paper may be useful in future studies on the mechanism of action of PLA_2 inhibitory proteins. All we can say at present is that the much higher PLA_2 inhibitory activity observed when P1 is pre-incubated with PLA_2 than with the lipid substrate, the apparent specificity of P1 for PLA_2, compared with phospholipase C and the very low inhibitor:substrate molar ratios at which PLA_2 inhibition is observed in our system indicate, at least for P1, a different mechanism of action from the 'lipid coating' model[30,31]. The construction of PLA_2-inhibitory oligopeptides from UG α-helix three and lipocortin I repeat three, on the basis of their high local similarity may indicate that similar sites of these proteins are responsible for their PLA_2 inhibitory activity. However, the region identified by our approach is not the only possible active site of lipocortins. We have not investigated whether either repeat two of lipocortin I, which may be necessary for the inhibitory activity[29], or lipocortin II, contain other PLA_2-inhibitory regions. However, when lipocortin I repeat three was aligned to lipocortin I repeat two and to lipocortin II repeat two by PRTALN, the region between residues 246-254 was aligned to positions 162-170 of lipocortin I and to positions 153-161 of lipocortin II.

Release and metabolism of arachidonate by mast cells are among the earliest biochemical changes in the inflammatory response to carrageenan[39]. They occur in response to carrageenan-induced 'cytoplasmic injury' of these cells[39]. Additionally, it has been suggested that liberation of PLA_2 from neutrophils participates in the amplification of the inflammatory response to carrageenan[39]. The *in vivo* pharmacological effects of PLA_2-inhibitory peptides make them a valuable model for the development of novel anti-inflammatory agents of therapeutic importance. Because the anti-inflammatory effect is the first of their biological properties to be identified, we propose the name 'antiflammins' for the peptides described here.

We thank Dr Jim Sidbury Jr for his support and suggestions Drs H. C. Chen and J. DeB. Butler for discussions and for critical review of the manuscript, and Dr Blake Pepinsky for the gift of recombinant lipocortin I. We wish to acknowledge the technical support provided by Dr Anita Hong and Mr Michael Brasseur (Applied Biosystems) and Dr Martha Knight (Peptide Biotechnologies, Inc.), who prepared the synthetic peptides used in this study.

Received 8 July, accepted 9 September 1988

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